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In re Application of: Stephen M. Allen Et Al.

Application No.: 10/659,199

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For: A NUCLIEC ACID ENCODING
A WHEAT BRITTLE-1 HOMOLOG

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tion that most of the ADP-Glc required for starch synthesis in maize endosperm is synthesized by cytosolic AGPase (Denyer et al., 1996), and that BT1 is the adenylate translocator responsible for the transfer of ADP-Glc into maize endosperm amyloplasts (Cao et al., 1995; Sullivan and Kaneko, 1995). Homologs of BT1 may be present in the amyloplast membranes from other starchy tissues, but they are not recognized by the antibodies to BT1 used by Cao et al. (1995) and by Cao and Shannon (1996, 1997).

Cytosolic localization of AGPase in maize endosperm cells is not supported by recent immunolocalization studies. For example, in a study using a transmission electron microscope, Miller and Chourey (1995) reported that proteins recognized by antibodies to spinach leaf AGPase were confined to amyloplasts, whereas antibodies to the peptide subunits of maize endosperm AGPase, BT2 and SH2, most heavily immunolabeled the amyloplasts and cell walls, with lighter labeling of the cytosol. In an in situ immunolocalization study at the light-microscopic level, Brangeon et al. (1997) observed that BT2 and SH2 antibodies (the same source of antibodies used by Miller and Chourey [1995]) immunolabeled both the amyloplasts and surrounding cytosol in pericarp cells from very young kernels, but immunolabel in endosperm cells from older kernels was closely associated with the amyloplasts only. These authors concluded that AGPase was localized in the amyloplast stroma of endosperm cells. However, at this level of resolution it is not possible to determine conclusively whether the immunolabeled proteins are in the plastid stroma or outside the envelope, and they correctly noted that the AGPase could have been bound to the outer membrane of the plastid envelope (Brangeon et al., 1997), and thus would partition as a "cytosolic" enzyme during aqueous fractionation.

A potential drawback of immunocytolocalization studies of cereal endosperm tissues at the electron-microscopic level is the difficulty encountered in sufficiently embedding the tissues so that the thin slices of starch granules do not "pop" out of the plastic before viewing. As a consequence, only amyloplasts with very small starch granules in cells located in the physiologically less-developed parts of the endosperm survive preparation for electron-microscopic examination. The surviving sections may or may not be representative of the entire tissue. Although this difficulty is minimized by using thicker sections for immunolocalization at the light-microscopic level, the resolution is not adequate to distinguish protein localization inside or outside of the plastid membranes.

There are also drawbacks to studies of enzyme compartmentation based on aqueously isolated amyloplasts. For example, during aqueous isolation most of the amyloplasts with starch granules larger than 1 or 2 μm in diameter are ruptured and the resulting preparation is enriched with amyloplasts containing smaller starch granules. To obtain the highest yield of intact amyloplasts, endosperms from very young kernels just beginning starch accumulation are used (Shannon et al., 1987). Activities of AGPase and SS were very low or undetectable in endosperms from 12-DPP kernels (Tsai et al., 1970), and Brangeon et al. (1997) showed a gradient of expression of the genes encoding AGPase from the periphery of the endosperm toward the center, with central endosperm cells of kernels 15 DPP most intensely immunolabeled by antibodies to BT2 and SH2. As a consequence, enzyme compartmentation in amyloplasts from very young kernels or from the physiologically younger cells near the periphery of the endosperm may not be representative of compartmentation in amyloplasts from those cells most actively engaged in starch biosynthesis.

To overcome these difficulties we developed two nonaqueous fractionation procedures to determine compartmentation of enzymes in amyloplasts from maize kernels in the linear phase of starch accumulation (about 20 DPP). Results of these studies are compared with results of an aqueous subcellular fractionation/immunoblotting study. Finally, results of a study of the uptake and incorporation into starch of metabolites by intact amyloplasts isolated from normal and mutant endosperms are reported. These studies support the conclusion that maize endosperm cells contain an extraplastidial form of AGPase, and that the amyloplast membrane-specific polypeptide, BT1, is an adenylate translocator.

MATERIALS AND METHODS

Plant materials were either grown in the field at the Russell E. Larson Agricultural Research Farm (Centre County, PA) or grown in 20-L plastic pots containing two parts peat, two parts perlite, and one part soil. Potted plants were grown in the greenhouse in late winter and spring or were started in the greenhouse in the spring and then transferred outside the greenhouse for continued growth. High-intensity sodium lamps were used in the greenhouse to extend the daylength to 16 h. Unless noted otherwise, the normal maize (*Zea mays* L.) inbred W64A and the endosperm mutant genotypes *waxy* (*wx*), *brittle-1* (*bt1*), and *shrunken-2* (*sh2*) in a near-isogenic W64A background were used in these studies.

Nonaqueous Compartmentation Studies

Estimating Amyloplast Compartmentation of Enzymes after Fractionation in Mixtures of TCE and Heptane

Endosperms from 20-DPP W64A inbred kernels were removed, frozen in liquid nitrogen, and freeze-dried. Pulverized samples were sifted through a 20-μm sieve using a sonic sifter fitted with a horizontal pulse generator (ATM Corp., Milwaukee, WI). The TCE/heptane procedure was patterned after methods used by Riens et al. (1991) and MacDougall et al. (1995). A total of 400 mg of dry, sifted endosperm in 50-mg batches was homogenized in 15-mL polypropylene centrifuge tubes in 2 mL of dry TCE using an ultrasonic probe (Biosonic IIA, Bronwill Scientific, Rochester, NY) for a total of 2.5 min using 30-s bursts. During homogenization the tube was held in a 95% ethanol/dry ice bath to reduce heating of the sample. Molecular sieve beads (4 Å, Sigma) were added to all TCE and heptane solutions before use to remove all traces of water, and

care was taken to keep all tubes tightly closed whenever possible.

The TCE homogenates were combined and *n*-heptane added to give a TCE:heptane mixture of 85:15 (v/v). Aliquots of this mixture were removed for "total-homogenate" analyses. The balance of the mixture was dispensed into several microcentrifuge tubes and the cellular contents were fractionated into amyloplast- and cytosol-enriched fractions by differentially pelleting the starch granules and associated enzymes from TCE/heptane mixtures of varying density. For example, the most dense fraction was pelleted from the 85:15 (v/v) TCE:heptane suspension by centrifugation in the cold (4°C) for 5 min at 16,000*g*. The pellet (pellet A) was retained and the supernatant was diluted with heptane to a final TCE:heptane ratio of 83:17 (v/v). Centrifugation was then repeated to yield pellet B. The resulting supernatant was again diluted with heptane to a ratio of 75:25 (v/v) and centrifuged as before to yield pellet C.

The 75:25 (v/v) TCE:heptane supernatant yielded the cytosol-enriched fraction. Aliquots of the initial unfractionated homogenate and the final cytosol-enriched supernatant were diluted with 3 volumes of heptane, and the particulate material in these heptane-diluted samples was collected by centrifugation in the cold for 10 min at 3000*g*. The clear supernatants were discarded and all pellets were held overnight at 4°C in a vacuum desiccator containing paraffin oil and silica-gel desiccant to remove the residual TCE and heptane. The dried pellets were extracted for enzyme analysis and the number of starch granules was determined. The TCE/heptane fractionation was repeated three times.

Amyloplasts in W64A endosperms each contain a single starch granule (Liu and Shannon, 1981); therefore, starch granule number was used as a measure of amyloplast number in the unfractionated homogenates and in the TCE/heptane fractions. We determined that the enzyme activities per million starch granules in the two most dense TCE/heptane fractions (pellets A and B) were very similar, and thus the means of both fractions ± SE (six values) were plotted. Likewise, activities per million starch granules in the unfractionated homogenate and in aqueous extracts of the sifted endosperm samples were very similar, and the means ± SE (six values) of these were plotted. The data from pellet C and supernatant fractions are the means ± SE of the three fractionations. To estimate compartmentation of an enzyme in amyloplasts, the average enzyme activity per million starch granules (*y* axis) from the four fractions (pellets A and B, unfractionated homogenate/aqueous extract, pellet C, and the supernatant) was plotted against the activity of a cytosol or vacuole marker enzyme per million starch granules from the same fractions (*x* axis). The *y* intersect of a regression line gives an estimate of enzyme activity per million amyloplasts in the absence of cytosol or vacuole contamination.

Glycerol Isolation of Amyloplasts

A procedure for the nonaqueous isolation of starch granules with associated metabolites (amyloplasts) from maize endosperm amyloplasts was reported previously (Liu and Shannon, 1981). In that procedure the dry endosperm sample was homogenized in dry glycerol and filtered through Miracloth (Calbiochem), and the starch granules were pelleted through a more dense solution of 3-Cl-1,2-propanediol. Although this procedure yielded an amyloplast fraction essentially free of nuclear and cytosolic contaminants, the starch biosynthetic enzymes were inactivated. We determined that inactivation was caused primarily by excessive heating of the sample during homogenization in glycerol and by exposure to 3-Cl-1,2-propanediol. The glycerol nonaqueous isolation procedure was therefore modified for the enzyme-compartmentation study. Fifty milligrams of sifted endosperm as used above was added to a microcentrifuge tube containing 1 mL of dry, cold (4°C) glycerol. The sample was thoroughly dispersed using a disposable plastic microtube pestle. The microcentrifuge tube was closed and placed on ice, and centrifugation was carried out at 4°C for 20 min at 25,000*g*. The supernatant was transferred to a 15-mL centrifuge tube and the pellet was washed with 0.5 mL of dry, cold glycerol by suspension using the microtube pestle and centrifugation as above. After the wash supernatant was added to the initial supernatant, the wall of the microcentrifuge tube was wiped with a tissue to remove excess glycerol. The combined supernatants and pellet were retained for enzyme analyses. Total enzyme activity in aqueous extracts of the sifted endosperm was also determined.

Enzyme Extraction and Assay

Duplicate samples of the TCE/heptane homogenate and of the four fractions were retained for enzyme analysis. Each fraction retained for extraction and enzyme assay was derived from approximately 42 mg of sifted endosperm. For the controls, duplicate subsamples (50 mg) of the sifted endosperm were also extracted and enzyme activities determined. Soluble enzymes were extracted from all pellet fractions and the sifted endosperm samples (TCE/heptane and glycerol) with 2 mL of HSB extraction buffer (50 mM Hepes, pH 7.5, 0.5 M sorbitol, 10 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM EDTA, 5 mM dithioerythritol, and 0.1% BSA) by sonication for four 10-s bursts with 10-s rest periods between each burst using the Biosonic IIA ultrasonic probe set at 60% maximum power. The tubes were held in an ice bath during sonication.

The glycerol supernatants (approximately 1.5 mL) were diluted to 5 mL with the HSB extraction buffer. The homogenates and diluted glycerol supernatants were centrifuged in the cold (4°C) for 10 min at 3000*g* and the supernatants retained for enzyme assay. Extracts from one set of the TCE/heptane pellets were used for assay of AGPase, UGPase, and ADH. AGPase and UGPase were assayed by the coupled-spectrometric method as described by Oh-Lee and Setter (1985), except that the AGPase and UGPase assays were started by the addition of 0.4 mM ADP-Glc and 0.4 mM UDP-Glc, respectively, and ADH as described by Cao et al. (1995). A small number of fractions were extracted at a time and AGPase, the most labile enzyme of the three, was assayed first. Extracts from the second set of

TCE/heptane pellets were used for assay of α-mannosidase as described by Boller and Kende (1979), for assay of APase as described by Gross and ap Rees (1986), and for assay of SBE and SS as described by Shannon et al. (1996). SUS was assayed in the hydrolytic direction as described by Echeverria et al. (1988), except that after heat inactivation, the quantity of Fru released was determined by a reducing sugar test as described previously (Shannon et al., 1996). The data were corrected for any Fru released in the absence of added UDP. HSB extracts of the glycerol fractions were used for assay of ADH, UGPase, AGPase, SBE, SS, and APase as described above.

Starch Granule Number

The number of starch granules remaining in the pellets after HSB extraction for enzyme assays was determined as described by Shannon et al. (1996).

Aqueous Compartmentation Study

Purification of Amyloplasts and Separation of Amyloplast Membranes and Stroma

Crude and Percoll-purified amyloplasts were isolated from the endosperm of freshly harvested developing kernels (13–16 DPP) as described by Cao et al. (1995). The purified amyloplast pellet was suspended in a small volume of TDEP buffer (10 mM Tricine, pH 7.2, 1 mM DTT, 1 mM EDTA, and 0.5 mM PMSF), and the amyloplasts were lysed by one cycle of freezing at −70°C and thawing at 30°C. After removal of starch granules by centrifugation at 800*g*, amyloplast stroma was separated from amyloplast membranes by centrifugation at 100,000*g* for 60 min. Amyloplast membranes were further purified from the crude membrane pellet through a discontinuous Suc-density gradient as described by Cao et al. (1995). The purified amyloplast membrane pellet was suspended in TDEP buffer plus 0.2 M Suc and stored at −70°C.

Isolation of Microsomal Membranes

Freshly isolated endosperms and other tissues were homogenized in a buffer containing 0.4 M Suc, 50 mM Mops, pH 6.9, 10 mM DTT, 1 mM EDTA, 0.1 mM PMSF, and 0.1% (w/v) BSA, and the homogenate was fractionated by differential centrifugation at 2,000*g* (P2), 10,000*g* (P10), and 100,000*g* (P100) according to the method of Cao et al. (1995). The P2 and P10 pellets and the microsomal membrane pellet (P100) were suspended in TES buffer (10 mM Tricine, pH 7.2, 1 mM EDTA, and 0.2 M Suc) and stored at −70°C.

Marker-Enzyme Analysis

SBE (an amyloplast marker) and ADH (a cytosol marker) were assayed as described above. Catalase (EC 1.11.1.6) (a marker for microbodies), Cyt *c* oxidase (EC 1.9.3.1) (a marker for mitochondria), cyanide-insensitive NADH-Cyt *c* reductase (EC 1.6.99.3) (a marker for the ER), and vanadate-sensitive ATPase (EC 3.6.1.4) (a marker for plasma membrane) were assayed as described by Cao et al. (1995). Potassium-stimulated ATPase (a marker for plasma membrane), Triton-stimulated UDPase (a marker for the Golgi), and nitrate-sensitive ATPase (a marker for the tonoplast) were assayed as described by Briskin et al. (1987). Protein contents were measured using the Bradford method plus NaOH treatment (Cao et al., 1995).

SDS-PAGE and Immunoblotting

Proteins were solubilized and denatured in 1× SDS gel-loading buffer by heating the samples in a boiling-water bath for 5 min. Polypeptides were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250 (Cao et al., 1995). Standard procedures were used for immunoblotting, as described previously (Cao et al., 1995). The polyclonal antibodies to maize SH2 and BT2 were gifts from Michael Giroux and L. Curtis Hannah, University of Florida (Giroux and Hannah, 1994), and the polyclonal antibodies to maize BT1 were a gift from Thomas D. Sullivan, University of Wisconsin-Madison (Sullivan and Kaneko, 1995). The relative quantities of BT1, SH2, and BT2 were determined by scanning densitometry (model 300B, Molecular Dynamics, Sunnyvale, CA) using a method similar to that described by Cao et al. (1995).

Calculation of the Cellular Localization of the BT2- and SH2-Antibody-Reacting Polypeptides of AGPase

The following data and calculations were used to determine the percentages of the SH2 and BT2 polypeptides of AGPase in the cytosol and amyloplasts. Total protein in the homogenate and crude amyloplast fraction (see Table III) was 3480 and 290 mg g^{-1} fresh weight, respectively. Thirty-one percent of the amyloplast marker enzyme (SBE) was recovered in the crude amyloplast fraction (amyloplast yield). The crude amyloplast fraction was contaminated with 0.7% cytosol (percentage of the cytosol marker enzyme ADH). Based on equal loading of proteins and as determined by immunoblotting analysis and densitometer scanning (see Fig. 4), we estimated that the crude amyloplast fraction contained 39% and 18% of the cellular SH2 and BT2 polypeptides, respectively.

To determine the percentage of SH2 compartmented in the cytosol, we set the homogenate (the crude amyloplast fraction [*A*] and the cytosol fraction [C]) containing 100% SH2 × 3480 mg of protein = 3480 SH2 units; and the SH2 in the crude amyloplast (*A* + 0.7% of C) fraction = 39% SH2 polypeptide × 290 mg of protein/31% (amyloplast yield) = 365 SH2 units. To solve for C: (*A* + C) − (*A* + 0.007*C*) = 3480 − 365; 0.993*C* = 3115; *C* = 3137 SH2 units and *A* = 3480 − 3137 = 343 SH2 units. Therefore, the percentage of SH2 in the cytosol = 3137/3480 × 100 = 90.1%. Based on a similar calculation we determined that the cytosol contained 95.8% of the cellular BT2 polypeptide, for an average of 93% of the cellular BT2- and SH2-antibody-reacting polypeptides of AGPase localized in the cytosol.

Metabolite-Uptake Studies

Radioactive Metabolites and Chemicals

Radioactive Glc-1-P, Glc-6-P, and ADP-Glc, uniformly ^{14}C labeled in the carbohydrate moiety, were purchased from ICN. Substrates, cofactors, inhibitors, and enzymes were obtained from Sigma, and all other chemicals used were analytical reagent grade.

Aqueous Amyloplast Isolation and Purification

Endosperms were removed from kernels 10 to 16 DPP (the precise ages are given in the tables) and homogenized in approximately 1 volume (w/v) of homogenization buffer (50 mM Hepes, pH 7.5, 0.5 M sorbitol, 10 mM KCl , 1 mM $MgCl_2$, 1 mM EDTA, 0.1% BSA, and 5 mM dithioerythritol) for 2 s at top speed in a homogenizer (VirTis 23, The VirTis Co., Gardiner, NY). The homogenate was gently filtered through Miracloth and an aliquot layered on a gradient of 10%, 20%, and 40% Percoll in the homogenization medium. The gradient was centrifuged for 5 min at 200*g* and the amyloplasts settling in the 20% Percoll layer were removed and used for the uptake studies. An aliquot of each preparation was removed to determine amyloplast intactness by measuring SBE activity before and after lysis, as described previously (Shannon et al., 1987).

Metabolite Uptake and Incorporation

For uptake and incorporation of ADP-Glc, amyloplasts (60–80 μL) were added to a reaction mixture (200 μL final volume) containing 100 mM Bicine, pH 8.5, 0.5 M sorbitol, 12.5 mM EDTA, 10 mM GSH, 50 mM $KC_2H_3O_2$, and 4 mM [^{14}C]ADP-Glc (the specific activity varied from 64 to 300 cpm/nmol). To determine the effect of ATP, ADP, or AMP on [^{14}C]ADP-Glc uptake and incorporation, the individual nucleotides (7 mM) were added to the amyloplasts in the 20% Percoll isolation buffer and incubated on ice for 30 min before an aliquot of the amyloplast suspension was added to the uptake medium (final nucleotide concentration in the uptake medium was 2.2 mM). To determine whether a translocator with an adenosine-binding site functions in the uptake of [^{14}C]ADP-Glc, the amyloplasts were preincubated for 30 min at 30°C in the uptake mixture containing varying concentrations of FSBA before the addition of [^{14}C]ADP-Glc. In the FSBA study each uptake solution contained 2% DMSO, the solvent for FSBA.

To determine the uptake and incorporation of Glc-1-P and Glc-6-P, amyloplasts (60–80 μL) were added to a reaction mixture (200 μL final volume) containing 15 mM Hepes, pH 7.5, 0.5 M sorbitol, 10 mM $MgCl_2$, 12.4 or 0.5 mM 3-PGA, 0.08% BSA, 0.1 unit of inorganic pyrophosphatase, and 2 mM [^{14}C]Glc-1-P (about 200 cpm/nmol) or [^{14}C]Glc-6-P (about 200 cpm/nmol). ATP at 2 mM and rabbit liver glycogen at 1 mg per uptake reaction were added as indicated.

All uptake studies were completed with intact amyloplast preparations and with lysed amyloplast preparations. There were no differences in the results when the amyloplasts were lysed either by including 1% Triton X-100 in the uptake medium or by brief sonication (four times for 10 s each, with cool-down periods between) of the uptake medium containing amyloplasts before the addition of the ^{14}C-metabolite. Unless noted otherwise the uptake reactions were carried out at 30°C and were terminated after 120 min by addition of 2 mL of 75% methanol containing 1% KCl. The alcohol-insoluble pellet was collected by centrifugation (2000*g*) in the cold for 10 min, and was washed twice with the methanol/KCl solution by suspension and centrifugation as above. The alcohol-washed pellets were then extracted three times with water by suspension and centrifugation as described above. The quantities of ^{14}C product in the water-soluble and -insoluble fractions were determined using a liquid-scintillation analyzer (Tri-Carb 1500, Packard Instrument Co., Downers Grove, IL).

An aliquot from each amyloplast isolation used for uptake studies was retained to determine the number of starch granules in each uptake reaction. Starch granule number was as determined previously (Shannon et al., 1996). Uptake and incorporation data are presented as the amount per million starch granules (amyloplasts). It is assumed that each amyloplast settling in the 20% Percoll layer contains one starch granule.

Protein-Sequence Analysis

The protein sequences used in the analysis were obtained from the literature and searched from the database of the National Center for Biotechnology Information. The locations of amino acid residues indicated in the tables correspond to the translated full-length sequences instead of the "mature" sequences. The sequence analysis was conducted as described previously for the alignment of branching enzymes (Cao and Preiss, 1996).

Transmission Electron Microscopy

Kernels were removed from 20-DPP *bt1* ears and small portions of endosperm were removed from the middle of the kernel and fixed for 4 h at room temperature in 4% glutaraldehyde in 100 mM cacodylate buffer, pH 7.0. The samples were postfixed for 1 h in 1% osmium tetroxide in the same buffer and then dehydrated through an ethanol series and embedded in the ultra-low-viscosity medium (VCD/HXSA) described by Oliveira et al. (1983). Silver to gold sections were cut using a diamond knife and a microtome (model III-8800, LKB, Bromma, Sweden) and examined by a transmission electron microscope (model 1200EXII, Jeol) either without additional staining or after staining with uranyl acetate and lead citrate.

RESULTS

Nonaqueous Fractionations

TCE/Heptane Fractionation

The percentage of recovery of starch granules and the activity of selected enzymes in the three fractions pelleting at various densities and the supernatant were all in excess of 80% of that in the original homogenate (Fig. 1). Forty-

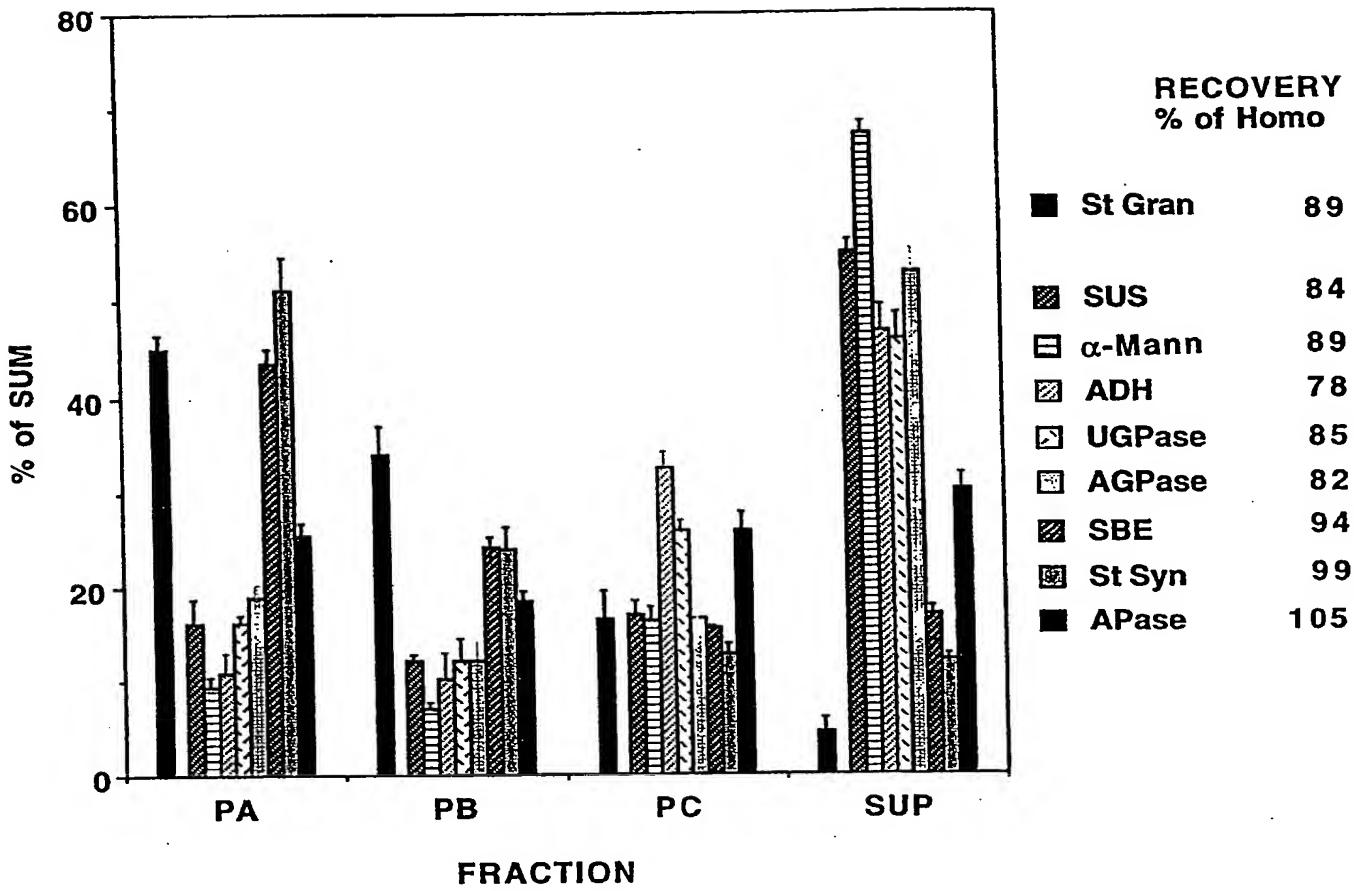

Figure 1. Distribution of starch granules and the activity of SUS, ADH, UGPase, AGPase, α-mannosidase (α-Mann), SBE, SS (St Syn), and APase in TCE/heptane fractions of different densities. The most dense fraction, pellet A (PA), was enriched in amyloplasts, and the least dense fraction, the supernatant (SUP) fraction, was enriched in cytosol. The distribution as a percentage of the sum of starch granules and enzyme activities from normal W64A endosperms and recovery of each as a percentage of the homogenate are recorded next to the figure key. Data are means ± SE of three fractionations. PB, Pellet B; PC, pellet C.

five percent of the starch granules was recovered in the most dense fraction (pellet A) and about 5% was recovered in the least dense fraction (the supernatant fraction). The amyloplast marker enzymes SBE and SS partitioned most closely with the starch granules, and the cytosol and vacuole marker enzymes, SUS, ADH, UGPase, and α-mannosidase, were low in the pellet A fraction and high in the supernatant fraction (Fig. 1). AGPase partitioned most closely with the cytosol marker enzymes and APase partitioned intermediate between the amyloplast and cytosol marker enzymes (Fig. 1).

The quantities of SBE, SS, APase, and AGPase associated with amyloplasts were determined from the *y* intercepts of the simple regression of the plot of target-enzyme activity per 10^6 starch granules versus nonplastid marker-enzyme activity per 10^6 starch granules. From this analysis we determined that 71%, 77%, and 58% of the putative amyloplast marker enzymes SBE, SS, and APase, respectively, were associated with the amyloplasts (Table I). The quantity of AGPase associated with the amyloplasts varied depending on the nonplastid marker used for the plot, but it is clear that little if any AGPase was recovered with the amyloplasts. These results are based on enzyme activities readily extracted in aqueous buffer solutions and are not expected to include the more tightly bound starch-granule-associated starch-synthase I and SBE II reported by Mu-Forster et al. (1996).

We intended to use the TCE/heptane procedure to estimate enzyme compartmentation in endosperm samples from the *bt1* mutant genotype. However, a critical part of the determination is an accurate count of the number of starch granules. We found that some of the TCE/heptane fractions contained a mixture of small starch granules (about 2 μm) and very small starch granules (less than 1 μm). Even though we stained the samples with iodine, we were unable to distinguish the smallest starch granules from protein bodies. In addition, the compartmentation calculation assumes that each amyloplast contains a single

Table I. *Compartmentation of enzymes in amyloplasts from W64A endosperm as determined using the TCE/heptane fractionation method*

SBE, SS, APase, and AGPase activities per million starch granules (*y* axis) in the TCE/heptane fractions were individually plotted against the activities per million starch granules of SUS, UGPase, ADH, and α-mannosidase (α-Mann), the nonplastidial marker enzymes. Estimates of SBE, SS, APase, and AGPase activities in amyloplasts per million starch granules were determined from the *y* intercept of a simple regression line from each individual plot. Activities of SBE, SS, APase, and AGPase per million starch granules in the total homogenate (Homo) are included. Data from three separate TCE/heptane fractionations were plotted.

Nonplastidial Enzyme	Activity in Amyloplasts			
	SBE	SS	APase	AGPase
		nmol min^{-1} 10^{-6} starch granules		
SUS	30.94	0.14	0.97	0.03
UGPase	27.80	0.14	0.65	−1.39
ADH	26.83	0.14	0.53	−1.84
α-Mann	33.17	0.15	1.17	1.00
Mean ± SE	29.68 ± 2.52	0.14 ± 0.01	0.83 ± 0.25	−0.55
		nmol min^{-1} 10^{-6} starch granules		
Homo activity	41.91 ± 2.20	0.19 ± 0.04	1.42 ± 0.06	4.15 ± 0.70
		% of Homo		
Amyloplast activity	70.8	76.9	58.4	0.0

starch granule. This is true for the normal inbred W64A and all maize endosperm mutant genotypes in the W64A background examined to date except for *su1* (Shannon and Garwood, 1984). However, the presence of the very small starch granules in the *bt1* samples caused us to question this assumption and we prepared fresh *bt1* endosperm samples for transmission electron microscopic examination. From this examination it is clear that endosperm cells from 20-DPP *bt1* kernels contain two populations of amyloplasts: simple amyloplasts with a single starch granule 1 to 5 μm in diameter, and compound amyloplasts, containing several very small starch granules (1 μm or less) (Fig. 2). Therefore, we were unable to accurately estimate enzyme compartmentation in *bt1* endosperm cells by the TCE/heptane procedure.

Nonaqueous Glycerol Isolation

The recovery of enzyme activity after the nonaqueous glycerol fractionation procedure varied between 40% and 117% of the enzyme activities measured after extraction in the HSB buffer (Table II). The glycerol-isolated amyloplast pellets contained only 8% and 7% of the cytosol marker enzymes, ADH and UGPase, respectively, and 14% of the AGPase activity (Table II). Thus, if we assume that the glycerol-isolated amyloplast pellet contains 7% cytosol contamination, then 7% of the cellular AGPase was compartmented in the amyloplasts. In contrast, 95%, 79%, and 38% of the recovered activities of the amyloplast marker enzymes SBE, SS, and APase partitioned with the glycerol-isolated amyloplasts, respectively. It is important to note that although the sum of APase activities in the glycerol supernatant and pellet fractions was 17% higher than in the HSB extract, only 56%, 40%, and 60% of the HSB-extractable activities of AGPase, SBE, and SS, respectively, were recovered in the glycerol-supernatant-plus-pellet fractions. The aliquots of HSB-diluted glycerol supernatant needed for assay of ADH, UGPase, and AGPase were smaller than those needed for assay of the plastid enzymes SBE and SS.

In a separate study we determined that the 0.3% to 1.5% of glycerol carried over from the diluted glycerol supernatant into the reaction mixtures was not inhibitory to ADH, UGPase, and AGPase, but that the 6% glycerol carried over into the assay mixtures for SBE and SS reduced measurable SBE and SS activities by approximately 60% and 25%, respectively, compared with assays in the absence of glycerol (data not shown). Thus, glycerol inhibition in the glycerol supernatants may contribute to the low recoveries of SBE and SS activities and inflate apparent partitioning of these enzymes in the glycerol-pellet fraction. When the percentage recoveries of the amyloplast marker enzymes associated with the glycerol pellets were calculated as percentages of the activity in the HSB extracts, we estimated that the glycerol pellets contained 38%, 47%, and 46% of the total cellular SBE, SS, and APase, respectively.

Activities of AGPase, SBE, SS, and APase per million starch granules in the glycerol-isolated amyloplasts (Table II) were very similar to the enzyme activities associated with the amyloplasts, as estimated by the TCE/heptane-fractionation procedure (Table I). It is clear from the results of these studies that a much higher percentage of AGPase partitions in the cytosol fraction compared with the amyloplast marker enzymes. In addition, we have shown that the nonaqueous glycerol procedure may be used to isolate starch-granule preparations from kernels in mid development (20 DPP), which contain almost half of the soluble stromal enzymes but are relatively free of cytosol marker enzymes.

Aqueous Fractionation and Immunolocalization

Preparation of Amyloplasts from Developing Maize Endosperm

As a second approach to confirm the subcellular localization of AGPase in maize endosperm, we isolated intact amyloplasts from 13-DPP endosperm. Typical examples of

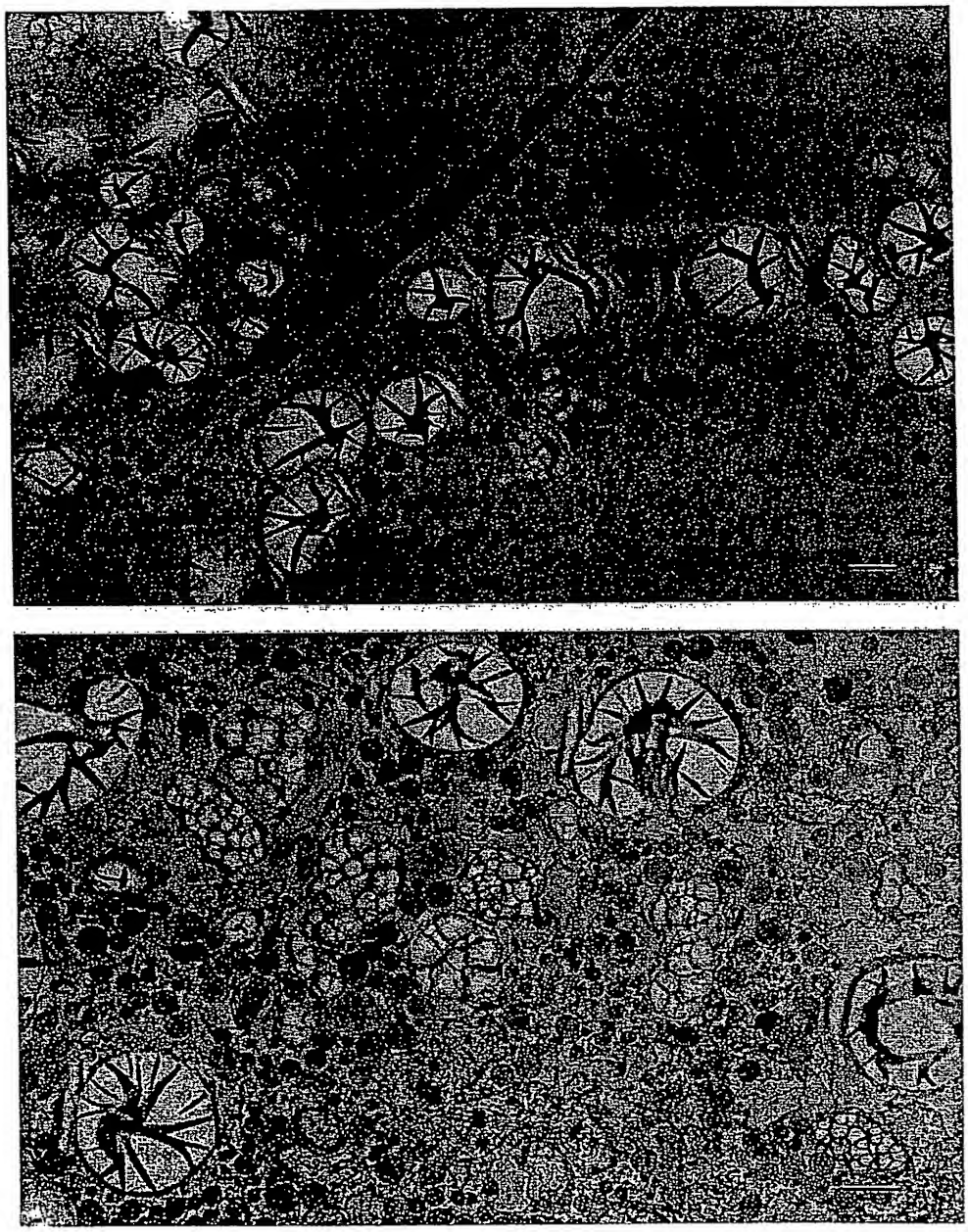

Figure 2. Low-magnification transmission electron photomicrographs of the parts of three cells in endosperm from a 20-DPP *bt1* mutant kernel. The section in the top micrograph was not poststained and that in the bottom micrograph was poststained with uranyl acetate and lead citrate. The large starch granules in the simple amyloplasts show dark artifacts that formed during sectioning because of the hydration and folding of the thin slices of starch. The compound amyloplasts contain many small starch granules. Bars = 2 μm.

the yield and purity of the aqueously isolated amyloplasts are summarized in Table III. Partially purified amyloplasts recovered in the 100*g* pellet (crude amyloplasts) contained approximately 31% of the amyloplast marker enzyme SBE and less than 1%, 4%, and 2% of the cytosol marker (ADH), the mitochondrial marker (Cyt *c* oxidase), and the ER marker (cyanide-insensitive NADH-Cyt *c* reductase) enzymes, respectively. Purification of the amyloplasts through a Percoll density gradient effectively removed all of the cytosol, mitochondria, and ER contaminants, but only 13.9% of the extractable SBE was retained with the more highly purified amyloplasts. Therefore, during Per-

Table II. *Partitioning of cytosol and amyloplast marker enzymes and AGPase in glycerol supernatant and pellet fractions*

Freeze-dried 20-DPP W64A endosperm tissues were pulverized and sifted through a 20-μm sieve. Samples of sifted endosperm were homogenized in glycerol (Gly) and separated into supernatant and pellet fractions. The activities in these fractions were compared with the total activities in subsamples of the sifted endosperm extracted in HSB buffer. Activities are presented per 50 mg of sifted endosperm sample and per million starch granules. Data are the average ± SE of the number of fractionations (shown in parentheses).

Enzyme	Fractionation	Enzyme Activity			Recovery after Gly	Activity in Gly Pellet	Activity in HSB or Gly Pellets
		Supernatant	Pellet	Total/sum			
		nmol min^{-1} 50 mg^{-1} dry wt			*% of HSB*	*% of sum*	*nmol min^{-1} 10^{-6} starch granules*
ADH (5)	HSB	–	–	1084 ± 202	–	–	9.77
	Gly-HSB	756 ± 80	67 ± 33	823 ± 93	76.0	8.2 ± 3.4	0.66
UGPase (4)	HSB	–	–	7935 ± 1860	–	–	71.49
	Gly-HSB	6457 ± 1009	483 ± 171	6940 ± 1055	87.5	7.0 ± 2.5	4.76
AGPase (5)	HSB	–	–	649.5 ± 108.7	–	–	5.85
	Gly-HSB	309.4 ± 72.4	51.2 ± 17.9	360.6 ± 88.3	55.5	14.2 ± 1.9	0.50
SBE (4)	HSB	–	–	6716 ± 920	–	–	60.51
	Gly-HSB	130 ± 59	2571 ± 187	2701 ± 228	40.2	95.2 ± 1.8	25.33
SS (4)	HSB	–	–	36.3 ± 6.5	–	–	0.33
	Gly-HSB	4.5 ± 0.6	17.2 ± 2.3	21.7 ± 2.9	59.8	79.3 ± 1.1	0.17
APase (6)	HSB	–	–	221.7 ± 39.2	–	–	1.99
	Gly-HSB	162.0 ± 32.3	98.4 ± 16.5	260.4 ± 44.3	117.5	37.9 ± 3.8	1.01

coll purification many of the amyloplasts were ruptured, releasing SBE from the amyloplasts. The crude and purified amyloplast preparations contained 8.3% and 3.4% of the homogenate protein, respectively, and the specific activity of SBE relative to that in the homogenate was enriched 3.7- and 4.1-fold in the crude and Percoll-purified amyloplast fractions, respectively (Table III).

Other cellular components such as the microbodies, plasma membrane, Golgi, and tonoplast cosedimented with the crude amyloplast preparation, resulting in a 2.2- to 5.5-fold increase in specific activity of these marker enzymes. However, after Percoll purification the amyloplast fraction was essentially free of catalase, the microbody marker, and contained only 0.6% of the cellular vanadate-sensitive ATPase, one of the markers for plasma membranes (Table III). The purified amyloplasts contained 5.9% of the cellular potassium-stimulated ATPase, a second putative plasma-membrane marker, but the specific activity had declined from 0.1 to 0.04. Likewise, the percentages of Triton-stimulated UDPase, a Golgi marker, and nitrate-sensitive ATPase, a tonoplast marker, were reduced to 4.2% and 3.5%, respectively, and their specific activities were much lower than in the crude pellet (Table III).

Membranes isolated from Percoll-purified amyloplasts were very yellow, with an absorption spectrum characteristic of carotenoids (plastid membrane marker): absorption peaks at 458 and 488 nm (data not shown). This membrane fraction was much enriched in the amyloplast membrane-specific polypeptide BT1 (Cao et al., 1995) compared with the total microsomal membranes (Fig. 3).

Table III. *Yield and purity of amyloplasts isolated from developing maize endosperm*

Amyloplasts were isolated from 13-DPP endosperm from cv Pioneer 3780 kernels. Aliquots were assayed for protein and marker enzymes after filtration through Miracloth (Homogenate), the first 100*g* centrifugation pellet (Crude Amyloplasts), and the 100*g* Percoll density-gradient-centrifugation pellet (Purified Amyloplasts). All samples were suspended in homogenization buffer and lysed by one freeze-and-thaw cycle before the starch was removed by centrifugation and the soluble protein content and marker enzyme activities were determined. Data are the average of two or three determinations. Homogenate protein is presented as milligrams per gram fresh weight and all enzyme results are presented as nanomoles per minute per gram fresh weight. Enzyme activities in the crude and purified amyloplasts are presented as a percentage of the activity in the homogenate (% of Homo) and as specific activity (Spec Act) nanomoles per minute per milligram protein.

Marker Enzyme	Compartment	Homogenate		Crude Amyloplasts		Purified Amyloplasts	
		Activity	Spec Act	% of Homo	Spec Act	% of Homo	Spec Act
Protein	–	3,480	–	8.3	–	3.40	–
SBE	Amyloplast	1,800	0.517	31.1	1.931	13.90	2.137
ADH	Cytosol	3,913	1.124	0.7	0.090	0	0
Cyt *c* oxidase	Mitochondria	69	0.020	3.6	0.009	0	0
NADH Cyt *c* reductase[a]	ER	1,113	0.320	1.7	0.064	0.02	0.002
Catalase	Microbodies	81,300	23.362	18.5	51.724	0.04	0.256
Vanadate-sensitive ATPase	Plasma membrane	143	0.041	18.3	0.090	0.60	0.008
Potassium-stimulated ATPase	Plasma membrane	74	0.021	39.9	0.102	5.90	0.038
Triton X-100-stimulated UDPase[b]	Golgi	121	0.035	46.6	0.194	4.20	0.044
Nitrate-sensitive ATPase	Tonoplast	76	0.022	33.6	0.088	3.50	0.023

[a] Cyanide-insensitive activity. [b] Color reagent without 1.5% SDS was used.

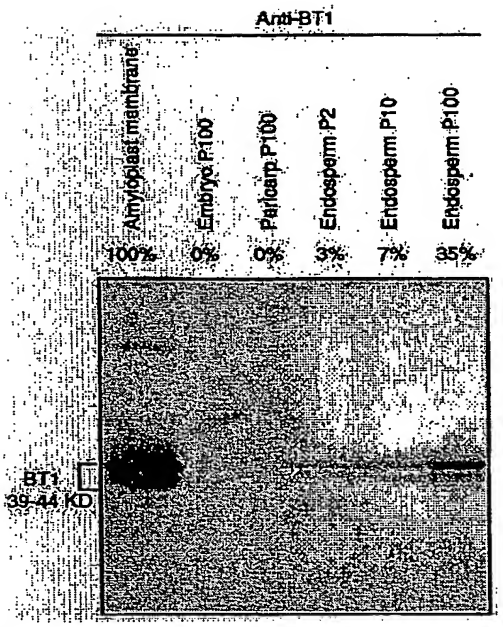

Figure 3. Immunolocalization of BT1 polypeptides in maize amyloplast membranes (lane 1), in microsomal membranes (P100) from the embryo, pericarp, and endosperm tissues (lanes 2, 3, and 6, respectively), and in pellets forming at 2,000*g* (P2) and 10,000*g* (P10) (lanes 4 and 5, respectively). The amyloplast membranes were isolated from endosperm amyloplasts purified from immature (approximately 12–15 DPP) Doebler 66XP hybrid kernels, and the P2, P10, and P100 fractions were isolated from 13-DPP Pioneer 3780 hybrid kernels as described previously (Cao et al., 1995). Polypeptides were separated by SDS-PAGE (15% separating gel), transferred to a nitrocellulose filter, and probed with polyclonal antibodies raised against a fusion protein containing 56 amino acids of the C terminus of BT1 and glutathione *S*-transferase (Sullivan and Kaneko, 1995). Lane 1 contained 8 μg of amyloplast-membrane protein; all other lanes contained 30 μg of protein. The relative quantities of BT1 in the various lanes as shown on the figure were determined by densitometry.

Immunolocalization of the BT2- and SH2-Antibody-Reacting Polypeptides of AGPase

Because the enrichment of amyloplasts based on the specific activity of SBE in the crude and Percoll-purified amyloplast preparations was similar but the yield of the amyloplast marker in crude amyloplasts was much higher than that in the purified amyloplast preparation, we chose crude amyloplasts for this experiment. Proteins from endosperm homogenates and crude amyloplasts were separated by 15% separating gel, transferred to nitrocellulose membranes, and probed with polyclonal antibodies raised against maize SH2 and BT2 polypeptides. The same-size polypeptides were detected in both the whole homogenate and the crude amyloplast preparations (Fig. 4).

The most significant result was that when equal quantities of protein were loaded, the intensities of SH2 or BT2 antibody-reacting polypeptides(s) were not enriched in the proteins from the crude amyloplast fraction relative to those in the homogenate (Fig. 4). Rather, based on densitometer analyses we estimated that the levels of SH2 and BT2 polypeptides in the crude amyloplasts were about one-third and one-fifth of those in the homogenate, respectively (Fig. 4). This lack of enrichment in the crude amyloplasts of the AGPase polypeptides was in sharp contrast to the approximately 4-fold enrichment of extractable SBE, the amyloplast stroma marker enzyme (Table III), and the 10-fold enrichment of the amyloplast membrane marker BT1 in amyloplast membranes recovered from the Percoll-purified amyloplasts (Fig. 3). This lack of BT2 and SH2 enrichment clearly indicates that the majority of the BT2- and SH2-antibody-reacting AGPase was localized outside of the amyloplasts.

Because the yield of amyloplasts in the crude amyloplast preparation was 31%, based on SBE activity, and cytosol contamination was 0.7%, based on ADH activity, we estimated that 90% and 95.8% of the total SH2 and BT2 proteins, respectively, or an average of 93% of the cellular BT2- and SH2-antibody-reacting polypeptides of AGPase, were located in the cytosol (see "Materials and Methods" for calculations). These values are very close to the estimates of AGPase compartmentation determined by the TCE/heptane and glycerol-isolation procedures reported above (Tables I and II).

Metabolite Uptake and Incorporation into Starch by Isolated Amyloplasts

Hexose-P Uptake and Incorporation

Intact amyloplasts were aqueously isolated and purified from normal and mutant endosperms and their capacities

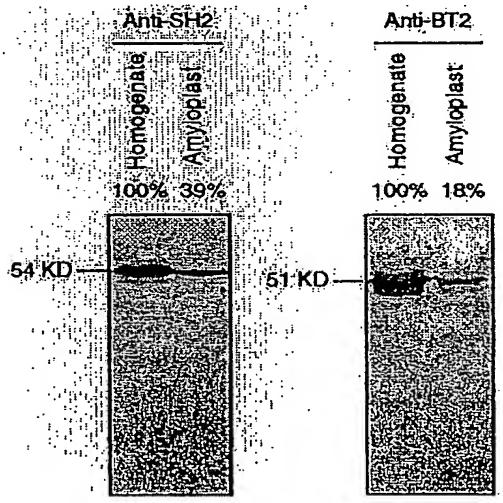

Figure 4. Immunolocalization of AGPase in the homogenate and in the crude amyloplasts (100*g* pellet) from maize endosperm cells. Polypeptides were separated by SDS-PAGE (15% separating gel), transferred onto nitrocellulose membranes, and probed with polyclonal antibodies raised against maize SH2 polypeptide (left) and maize BT2 polypeptide (right) (Giroux and Hannah, 1994). All lanes contained 12 μg of protein. Lanes 1 and 2 contained peptides from the homogenate and the crude amyloplasts, respectively. The relative quantity of SH2 and BT2 protein shown on the figure was determined by densitometry.

for the uptake and use of hexose-Ps and ADP-Glc for starch synthesis were determined. Sixty percent or more of the amyloplasts used for these studies were judged to be intact based on latency analysis (data not shown). Table IV summarizes the incorporation of [^{14}C]Glc into a methanol-insoluble product after incubation of intact or lysed amyloplasts in uptake medium containing [^{14}C]Glc-1-P or [^{14}C]Glc-6-P either with or without added ATP. In the absence of added glycogen, very little [^{14}C]Glc from either hexose-P was incorporated into the methanol-insoluble product. Generally, Glc transfer from Glc-1-P was somewhat higher than that from Glc-6-P, but there was little if any effect of added ATP, a substrate for AGPase. In addition, incorporation by amyloplast from the AGPase-deficient mutant *sh2* was equal to that by amyloplasts from normal, *wx* and *bt1* endosperms.

The similarity in incorporation of [^{14}C]Glc-1-P by all genotypes tested and the small effect of added ATP indicates that polymerization was most likely caused by the activity of starch phosphorylase, with little contribution by plastid-localized AGPase. The standard uptake solution used in these studies contained relatively high levels of 3-PGA (12.4 mM), the allosteric activator of AGPase. It is possible that the low incorporation of hexose-Ps was the result of 3-PGA inhibition of hexose-P uptake, but this was ruled out by a later study in which we showed that uptake and incorporation of hexose-Ps in the presence of 0.5 and 12.4 mM 3-PGA were similar (Table IV).

During incubation some of the amyloplasts are invariably ruptured, releasing plastid enzymes. The consequences of this were seen when glycogen, an alternative glucan acceptor, was added to the "intact" and lysed amyloplasts incubated with Glc-1-P. Over 10 times more [^{14}C]Glc was incorporated into the methanol-insoluble polymer but, again, there was little if any effect of added ATP (Table IV). This supports the conclusion that the isolated amyloplasts contain an active starch phosphorylase that effectively transfers Glc from Glc-1-P to glycogen, but was much less effective in Glc transfer to the native glucan acceptors (starch granules) of the amyloplasts. Apparently, amyloplasts isolated from *sh2* (the only genotype tested) contain very little phosphoglucomutase, or that which is present is essentially inactive in the uptake conditions used, because even in the presence of added glycogen there was very little transfer of [^{14}C]Glc from Glc-6-P to the methanol-insoluble product (Table IV).

ADP-Glc Uptake and Incorporation

Intact maize amyloplasts isolated from 10- to 16-DPP normal, *wx*, and *sh2* endosperms incorporated more than 10 times as much Glc from ADP-Glc into a methanol- and water-insoluble product (Table V) as from Glc-1-P (Table IV). This difference in incorporation was not caused by a difference in the buffer salt or pH of the uptake solutions used in the standard conditions, because in a later study we determined that there was no difference in Glc incorporation from ADP-Glc when the uptake solution was buffered with Hepes at pH 7.5 rather than at pH 8.5 (Table V). Hydrolysis of the water-insoluble product with β-amylase yielded maltose and the product was judged to be starch (data not shown). Lysis of the amyloplasts before incubation reduced incorporation 70% to 90%. Among the three genotypes, intact amyloplasts from *sh2* most effectively converted ADP-Glc to starch. In contrast, intact amyloplasts from the other starch-deficient/high-sugar genotype, *bt1*, was only 26% as effective in the uptake and conversion of ADP-Glc to starch as amyloplasts from *wx*,

Table IV. *Hexose-P uptake and incorporation into methanol-insoluble product*

Amyloplasts were from the 20% Percoll fraction. The reaction mixture (200 μL final volume) contained 15 mM Hepes, pH 7.5, 0.08% BSA, 10 mM $MgCl_2$, 12.4 mM 3-PGA, 0.5 M sorbitol, 0.1 unit of inorganic pyrophosphatase, 2 mM Glc-1-P or Glc-6-P, 2 mM ATP, and 1 mg of rabbit-liver glycogen (RLG) as indicated plus 60 or 80 μL of amyloplast fraction. For the uptake studies amyloplasts were isolated from the endosperms of the following age kernels: N, 12 DPP; *wx*, two 12 DPP, one 13 DPP, and one 16 DPP for the minus-RLG study, and one 13 DPP and one 16 DPP for the plus-RLG study; *sh2*, one each of 13, 15, and 16 DPP; and *bt1*, one 11 DPP.

Genotype (*n*)	Total Incorporation							
	Glc-1-P				Glc-6-P			
	Intact		Lysed		Intact		Lysed	
	+ATP	−ATP	+ATP	−ATP	+ATP	−ATP	+ATP	−ATP
	pmol min^{-1} 10^{-6} amyloplasts							
No RLG								
N (1)	4.17	1.83	2.08	–	–	–	–	–
wx (4)	4.54 ± 0.50	3.90 ± 1.42	4.12 ± 1.22	–	1.96 ± 1.37	1.25 ± 0.42	1.08	–
wx (1)[a]	6.50	–	1.36	–	7.11	–	1.12	–
sh2 (3)	5.11 ± 0.58	3.87 ± 1.67	2.81 ± 0.75	1.52 ± 1.00	0.83 ± 0.14	0.60 ± 0.21	0.44 ± 0.12	0.72 ± 0.68
bt1 (1)	5.67	4.83	4.50	–	6.08	1.92	1.58	–
Plus RLG								
wx (2)	43.75 ± 6.1	37.78 ± 7.3	63.94 ± 0.7	58.49 ± 1.1	–	–	–	–
sh2 (2)	–	–	47.56 ± 9.1	42.93 ± 9.1	–	–	2.49 ± 0.7	2.02 ± 0.6

[a] For this experiment the uptake solution contained 0.5 mM 3-PGA rather than the 12.4 mM used for all of the others.

Table V. *ADP-Glc uptake and incorporation into methanol- and water-insoluble products*

Amyloplasts were from the 20% Percoll fraction. The reaction mixture (200 μL final volume) contained 100 mM Bicine, pH 8.5, 0.5 M sorbitol, 12.5 mM EDTA, 10 mM GSH, 50 mM potassium acetate, and 4 mM [^{14}C]ADP-Glc plus 60 or 80 μL of amyloplast fraction. For the uptake studies amyloplasts were isolated from the endosperms of the following age kernels: N, two 10 DPP, one 11 DPP, and two 12 DPP; *wx*, one 10 DPP, three 12 DPP, five 13 DPP, and one 14 DPP; *sh2*, one each of 15 and 16 DPP; and *bt1*, one each of 11, 12, and 14 DPP. Data are averages ± SE.

Genotype (*n*)	Total Incorporation		Percentage Water Insoluble	
	Intact	Lysed	Intact	Lysed
	pmol min^{-1} 10^{-6} amyloplasts		*% of total methanol insoluble*	
N (5)	58.58 ± 24.5	12.33 ± 7.75	90 ± 6	87 ± 8
wx (10)	67.25 ± 16.83	6.17 ± 4.00	79 ± 6	80 ± 11
wx (1)[a]	49.37	6.29	94	95
sh2 (2)	133.75 ± 8.42	42.92 ± 3.67	86 ± 10	92 ± 4
bt1 (3)	18.00 ± 6.17	12.33 ± 4.17	67 ± 4	87 ± 1

[a] For this experiment the uptake solution contained 15 mM Hepes, pH 7.5, 0.08% BSA, 10 mM $MgCl_2$, 0.5 M sorbitol, and 4 mM ADP-Glc plus 80 μL of amyloplast suspension.

but incorporation by lysed *bt1* amyloplasts was similar to that by lysed normal and *wx* amyloplasts.

The addition of glycogen, an alternative glucosyl acceptor, to the reactions containing the lysed amyloplasts from *wx* and *bt1* genotypes restored incorporation equal to or greater than that in reactions containing intact *wx* amyloplasts (Table VI). Thus, the reduced [^{14}C]Glc incorporation from ADP-Glc into starch by *bt1* amyloplasts was caused by the reduced transfer of ADP-Glc into the amyloplasts. In addition, we can conclude from these results that the reduced incorporation from ADP-Glc by lysed amyloplasts in the absence of added glycogen acceptor was apparently the result of dilution of the SS relative to the nonreducing ends of the native maltooligosaccharide or starch-granule acceptors. Enhanced incorporation of [^{14}C]Glc from ADP-Glc in the presence of rabbit-liver glycogen (Table VI) indicates that glycogen may be a better substrate for SS than the native acceptors.

If an adenylate translocator in the amyloplast membrane of the isolated intact amyloplasts is functioning in the uptake of ADP-Glc in exchange for ADP or AMP, then these ADP or AMP nucleotides in the uptake medium might compete with ADP-Glc for uptake. In fact, preincubation of the amyloplasts in the cold for 30 min in the presence of 7 mM ADP or AMP, followed by [^{14}C]ADP-Glc uptake and incorporation in the presence of 2.2 mM ADP or AMP, reduced ^{14}C incorporation into starch by 75% and 82%, respectively (data not shown). SS synthesis activities, as measured in reactions containing lysed amyloplasts plus glycogen, were inhibited 60% and 63%, respectively, by these ADP and AMP treatments. Therefore, in this study we were unable to distinguish between the effects of the nucleotides on ADP-Glc uptake and their effects on SS activity.

The adenosine analog FSBA is well known to react with adenosine nucleotide-binding sites of enzymes and proteins (Colman, 1983), including mitochondrial F_1-ATPase (Esch and Allison, 1978), chloroplast ATPase (DeBenedetti and Jagendorf, 1979), and an ADP-binding protein on the exterior surface of human platelets (for review, see Colman, 1983). If ADP-Glc is transported into amyloplasts via an adenylate translocator, then we predicted that FSBA would inhibit ADP-Glc uptake into intact amyloplasts, resulting in reduced incorporation of Glc into starch. To test this we pretreated intact amyloplasts from *wx* in the standard uptake medium containing 0 to 4 mM FSBA dissolved in DMSO. All pretreatment and uptake solutions contained 2% DMSO (the FSBA solvent), which had no negative effect on uptake of ADP-Glc and incorporation of [^{14}C]Glc into the methanol-insoluble product in either the absence or presence of rabbit-liver glycogen (incorporation in the presence of glycogen is a measure of SS activity) (Table VII).

Inhibition of uptake and incorporation by intact amyloplasts increased with increasing concentrations of FSBA. FSBA would also be expected to interact with the ADP-Glc-binding site of SS, but at 2 and 4 mM FSBA, reduction in uptake and incorporation into starch by intact amyloplasts was greater than the inhibition of SS as measured in lysed Table VII amyloplasts in the presence of added glycogen (Table VII). For intact amyloplasts incubated in the absence of glycogen (except at the highest FSBA treatment), almost three-fourths of the methanol-insoluble radioactivity was incorporated into the water-insoluble starch granules. The amyloplast-uptake studies provide evidence that cytosolic synthesized ADP-Glc can be transferred across amyloplast membranes via an adenylate translocator. We have proposed that BT1 is that adenylate translocator in maize endosperms (Shannon et al., 1996).

Table VI. *Effect of glycogen on ADP-Glc incorporation into methanol-insoluble products*

Amyloplast preparation and uptake conditions were the same as in Table V, with the addition of 1 mg of rabbit-liver glycogen (RLG) where noted. For each genotype, data are the means of duplicate incubations of a single preparation of amyloplasts from 13 DPP *wx* endosperm and from 14 DPP *bt1* endosperm.

Genotype	Total Incorporation		
	Intact	Lysed	Lysed + RLG
	pmol min^{-1} 10^{-6} amyloplasts		
wx	78.2	13.6	110.3
bt1	26.5	18.1	130.9

Table VII. *FSBA inhibition of ADP-Glc uptake and incorporation into methanol- and water-insoluble products and inhibition of SS*

Amyloplasts in the 20% Percoll fraction isolated from 13 DPP *wx* endosperms were used. The amyloplasts were preincubated for 30 min at 30°C in their respective reaction mixtures before the addition of [^{14}C]ADP-Glc. The reaction mixtures were as described in Table VI, with the addition of 2% DMSO and FSBA as noted. Reaction mixtures containing 1 mg of rabbit-liver glycogen (RLG) provide a measure of SS activity. Control intact and lysed amyloplasts, without DMSO, incorporated 34.62 and 4.63 pmol min^{-1} 10^{-6} amyloplasts, respectively.

FSBA	RLG	Total Incorporation of [^{14}C]ADP-Glc		Inhibition of Incorporation by FSBA		Incorporated into Starch Granules	
		Intact	Lysed	Intact	Lysed	Intact	Lysed
mM		pmol min^{-1} 10^{-6} amyloplasts				%	
0	−	48.25	5.67	0	0	76	92
1	−			6	0	68	97
2	−			43	33	69	67
4	−			62	30	53	54
0	+	57.92	59.08	0	0	27	8
1	+			0	9	37	10
2	+			3	16	30	8
4	+			20	41	25	10

Identification of a Putative ADP-Glc-Binding Motif in BT1

If BT1 is the adenylate translocator functioning in the transfer of ADP-Glc into amyloplasts, then BT1 must contain an ADP-Glc-binding motif. Analysis of the full-length BT1 sequence showed the presence of a KTGGL motif. This motif was identified as the ADP-Glc-binding site of *Escherichia coli* glycogen synthase (Furukawa et al., 1993) and is conserved in all known enzymes that use ADP-Glc as a substrate, including plant SS and bacterial glycogen synthases (Table VIII). The KTGGL motif in BT1 is 40 amino acid residues upstream of the transit-peptide cleavage site proposed by Sullivan et al. (1991). Thus, if this proposed ADP-Glc-binding motif is present in the mature BT1 protein, then an alternative transit-peptide cleavage site is required. Comparison of several known N-terminal sequences of SS revealed a consensus cleavage site of V(I)X/A(G,S), and in BT1 an alternative cleavage site, VP/A, is present 13 amino acid residues upstream of the KTGGL motif, the proposed ADP-Glc-binding site (Table IX). Cleavage at this site would yield a mature BT1 protein of 44

Table VIII. *Positions of ADP-Glc-binding motifs in the full-length BT1 protein and in various starch (SS) and glycogen synthases (GS)*

Protein	Accession No.	Motif	Position	Reference
Maize BT1	M79333	KTGGL V A S	35–39	Sullivan et al. (1991)
ADP-Glc-binding consensus sequence		KTGGL		
Barley WX	X07932	KTGGL	90–94	Rohde et al. (1988)
Cassava WX	X74160	KTGGL	96–100	Salehuzzaman et al. (1993)
Maize WX	M24258	KTGGL	90–94	Kloesgen et al. (1986)
Pea GBSSI	X88789	KTGGL	91–95	Dry et al. (1992)
Pea GBSSII	X88790	KTGGL	255–259	Dry et al. (1992)
Potato SS	X87988	KTGGL	360–364	Edwards et al. (1995)
Potato SSIII	X94440	KVGGL	794–798	Abel et al. (1996)
Potato SSSI	Y10416	KTGGL	145–149	(G.I.W. Abel, J. Kossman, and L. Willmitzer, unpublished data)
Potato SSSIII	X95759	KVGGL	794–798	Marshall et al. (1996)
Potato WX	X58453	KTGGL	95–99	van der Leij et al. (1991)
Rice SSS	D16202	KSGGL	97–101	Baba et al. (1993)
Rice WX	X62134	KTGGL	97–101	Okagaki (1992)
Sorghum WX	U23945	KTGGL	96–100	Hsieh et al. (1996)
Sweet potato SS	U44126	KTGGL	98–102	(S.-J. Wang, K.W. Yeh, and C.-Y. Tsai, unpublished data)
Wheat WX	X57233	KTGGL	91–95	Clark et al. (1991)
E. coli GS	J02616	KTGGL	15–19	Kumar et al. (1986)
Bacillus stearothermophilus GS	D87026	KSGGL	15–19	(H. Takata, T. Takata, S. Okada, M. Takagi, and T. Imanaka, unpublished data)
Bacillus subtilis GS	Z25795	KSGGL	15–19	Kiel et al. (1994)
Agrobacterium tumefaciens GS	L24117	KTGGL	15–19	Uttaro and Ugalde (1994)
Synechocystis GS	D90899	KAGGL	15–19	Kaneko et al. (1996)

Table IX. *Positions of putative transit peptide cleavage sites in the full-length BT1 protein and in various starch synthases (SS)*

Protein	Accession No.	Cleavage Site	Method	Position	Ref.
Maize BT1	M79333	SLQVP/AV· S	Sequence alignment	24/25	Sullivan et al. (1991)
Transit peptide cleavage site Consensus sequence		I G VX/A			
Barley WX	X07932	SVVVS/AT	Sequence alignment	70/71	Rohde et al. (1988)
Cassava WX	X74160	AKIIC/GH	Sequence alignment	78/79	Salehuzzaman et al. (1993)
Maize WX	M24258	SLVVC/AS	Protein sequencing	72/73	Kloesgen et al. (1986)
Pea GBSSI	X88789	GKIVC/GM	Protein sequencing	75/76	Dry et al. (1992)
Pea GBSSII	X88790	KQHVR/AV	Protein sequencing	57/58	Dry et al. (1992)
Potato SS	X87988	NQRVK/AT	Protein sequencing	65/66	Edwards et al. (1995)
Potato WX	X58453	ATIVC/GK	Protein sequencing	77/78	van der Leij et al. (1991)
Rice SSS	D16202	TIFVA/SE	Protein sequencing	24/25	Baba et al. (1993)
Rice WX	X62134	SVVVY/AT	Protein sequencing	77/78	Okagaki (1992)
Sorghum WX	U23945	SLVVC/AT	Sequence alignment	77/78	Hsieh et al. (1996)
Wheat WX	X57233	SMVVR/AT	Protein sequencing	70/71	Clark et al. (1991)

kD, which agrees well with the 39 to 44 kD for BT1 reported previously (Cao et al., 1995).

DISCUSSION

In this paper we summarize the results of several studies that strongly support the conclusion that in maize endosperm most of the cellular AGPase is localized in the cytosol, and that the inner amyloplast-membrane-specific polypeptide, BT1, is an adenylate translocator that functions in the transfer of cytosol-synthesized ADP-Glc into the amyloplasts. Denyer et al. (1996) reported that more than 95% of AGPase activity in maize endosperm cells is extraplastidial. This result was based on aqueous fractionation of endosperm homogenates from young (11–17 DPP) kernels. During fractionation approximately 75% of the amyloplast marker enzymes were lost from the amyloplast fraction (Denyer et al., 1996), and we know from experience that a greater proportion of the more mature amyloplasts with the larger starch granules are lysed during isolation. Consequently, the final preparation would be enriched with amyloplasts containing small starch granules. Enzyme compartmentation in such an amyloplast preparation may not be representative of compartmentation in the more mature amyloplasts from cells in the linear phase of starch accumulation.

This concern is validated by a recent immunolocalization study by Brangeon et al. (1997), which clearly shows that the peripheral endosperm cells were only lightly immunolabeled by antibodies to BT2 and SH2 and that there was a gradient of increasing signal intensity that paralleled the increase in starch-granule size. We have developed and used nonaqueous TCE/heptane fractionation and nonaqueous glycerol-isolation methods to show for the first time, to our knowledge, that in the more mature maize endosperm cells (20 DPP), 90% or more of the cellular AGPase is cytosolic (Tables I and II). These studies were based on the observation that during freeze-drying, much of the amyloplast stromal content dries onto the surface of the starch granule, and the starch granule also shrinks away from the cytosol (Liu and Shannon, 1981). Therefore, during nonaqueous fractionation (Table I) or isolation (Table II) the stromal enzymes associated with the starch granules remain with the granules until they are extracted with the aqueous buffer solution.

With the TCE/heptane protocol, patterned after the procedures used by Riens et al. (1991) and MacDougall et al. (1995), pulverized endosperm samples were separated into several fractions with varying enrichments in amyloplasts or cytosol. With this procedure enzyme compartmentation in amyloplasts was determined from plots of the activity of the enzyme in question per million starch granules against the activity of nonplastidial enzymes per million granules. This procedure has the advantage that enzyme recovery was high after TCE/heptane fractionation. It was clear from these data that AGPase closely partitioned with the nonplastidial marker enzymes (Fig. 1), and 58% to 77% of the plastid marker-enzyme activities were retained with the starch granules (Table I).

Although the TCE/heptane procedure could be used to estimate enzyme compartmentation in amyloplasts, it was not satisfactory for the isolation of a cytosol-free starch-granule preparation with associated stromal enzymes (amyloplasts). To accomplish this we modified the glycerol-based procedure of Liu and Shannon (1981). The resulting starch-granule preparations, which were contaminated with about 7% of the cytosol marker enzymes, retained approximately 14% of the cellular AGPase (7% more than cytosolic contamination) and 50% of the amyloplast marker-enzyme activities (Table II). It is significant that enzyme activities per million starch granules determined by both nonaqueous procedures were approximately the same (Tables I and II). Results of the nonaqueous studies that demonstrated predominant cytosolic localization of AGPase were confirmed by immunolocalization of BT2 and SH2 polypeptides in aqueously isolated amyloplasts (Fig. 4).

Earlier studies of compartmentation of AGPase in maize endosperm cells have been controversial. In contrast to the extra-amyloplastic localization of AGPase reported by

Denyer et al. (1996), results of immunocytolocalization studies have been interpreted as showing that most, if not all, AGPase is localized in the amyloplasts (Miller and Chourey, 1995; Brangeon et al., 1997). Both approaches to the study of compartmentation in maize endosperm have serious disadvantages. First, as noted above, 75% or more of the amyloplasts are ruptured during aqueous isolation, resulting in an amyloplast preparation enriched in plastids containing the smaller starch granules (Shannon, 1987). Second, a serious drawback of immunocytolocalization studies of maize endosperm at the electron-microscopic level is the difficulty of sufficiently embedding the tissue so that the thin slices of starch granules remain in the plastic. Consequently, regions of the endosperm consisting of cells with small starch granules are more likely to survive preparation. In maize endosperms such cells occur in very young kernels (about 12 DPP) or in the peripheral cells.

As noted above, enzyme compartmentation in these physiologically less mature cells may not be representative of compartmentation in cells more actively engaged in starch synthesis (Tsai et al., 1970; Brangeon et al., 1997). In addition, it is possible that cytosolic enzymes may be lost from the tissue piece during preparation of the samples for electron-microscopic examination; in fact, Miller and Chourey (1995) pointed out that they were unable to immunolocalize the cytosol-specific enzyme SUS. Tissue preparation and cutting of the thicker sections suitable for immunocytolocalization at the light-microscopic level are less problematic than preparation for electron-microscopic studies. In a light-microscopic immunolocalization study of AGPase compartmentation in maize kernels, Brangeon et al. (1997) clearly showed that in pericarp cells of kernels 8 DPP, polypeptides recognized by antibodies to the AGPase subunits BT2 and SH2 were cytosolic, but in endosperm cells actively engaged in starch synthesis (15 DPP), the antibodies immunolabeled only polypeptides that were closely associated with amyloplasts. These authors suggested an intraplastidial localization for the AGPase polypeptides encoded by *Bt2* and *Sh2* in these maize endosperm cells. However, they suggest that at this level of resolution, it is not possible to distinguish between proteins in the amyloplast stroma and proteins located either between the inner and outer membranes of the amyloplast envelope or closely associated (loosely bound) with the outer membrane (Brangeon et al., 1997).

The compartmentation results obtained in the present study using nonaqueous procedures do not support the suggestion of Brangeon et al. (1997) that AGPase is localized in the amyloplast stroma. Rather, we show that AGPase resides in a compartment that partitions with the cytosol during nonaqueous fractionation. However, because the soluble enzymes located within the inner-membrane space of the amyloplast envelopes and those in close association with the amyloplasts in situ would be expected to partition with the cytosol during aqueous and nonaqueous fractionation or isolation, we were unable to rule out the possibility that AGPase resides within the inner-membrane space of amyloplasts.

BT1 Is an Adenylate Translocator

Two phosphate translocators (Fischer et al., 1997; Kammerer et al., 1998) and two adenylate translocators (Möhlmann et al., 1997) have been reported to be present in maize endosperm amyloplast membranes. Fischer et al. (1997) isolated and characterized a PEP/Pi antiporter that is present in plastid membranes from both photosynthetic and nonphotosynthetic tissues. The Glc-6-P/Pi antiporter was shown to be preferentially expressed in nonphotosynthetic tissues (Kammerer et al., 1998) and to mediate the 1:1 exchange of Glc-6-P with Pi and triose phosphate, and is assumed to function in vivo in the import of Glc-6-P into amyloplasts. Kammerer et al. (1998) suggest that Glc-6-P may be used either in the starch biosynthetic pathway or as a substrate for the oxidative pentose-phosphate pathway. Neuhaus et al. (1993) reported the isolation of amyloplasts from maize endosperm that were capable of uptake and incorporation Glc-6-P into starch. Möhlmann et al. (1997) used a similar amyloplast-isolation procedure and determined that Glc from ADP-Glc was incorporated into starch at a rate 6 times higher than that from Glc-6-P.

The amyloplast-isolation procedure used for these studies, which included multiple high-speed centrifugations through density gradients, yielded a preparation of amyloplasts with very small starch granules (Neuhaus et al., 1993) and most likely also contained amyloplast membrane vesicles without starch granules. We have used a much more gentle amyloplast-isolation procedure, and the results of the hexose-P uptake and incorporation studies presented in this paper do not support the use of Glc-6-P in the starch biosynthetic pathway (Table IV). Intact amyloplasts from maize endosperm were relatively inefficient in the uptake and conversion of Glc-1-P and Glc-6-P into starch regardless of whether ATP was included in the uptake medium. In this study we measured incorporation of radioactive hexoses into starch and did not attempt to determine hexose-P uptake independent of its utilization in starch synthesis. If we assume that the amyloplast membranes contain a functional Glc-6-P/Pi antiporter, then the imported Glc-6-P is a poor substrate for starch synthesis. The amyloplasts used for these uptake and incorporation studies were isolated from young kernels that may not have developed their full complement of AGPase activity (Tsai et al., 1970; Brangeon et al., 1997), and this may have contributed to the poor utilization of hexose-Ps. However, when considering the predominant cytosolic localization of AGPase (Table I and II), the poor hexose-P utilization may simply reflect the minor role of amyloplastic AGPase in starch synthesis.

Amyloplasts isolated from *wx* and *sh2* endosperms apparently do contain active starch phosphorylase, because when glycogen, an alternative glucan acceptor, was included in the uptake mixture Glc was effectively transferred from Glc-1-P to the glycogen acceptor (Table IV). In contrast, Glc-6-P was a poor substrate for Glc addition to glycogen, indicating either that the amyloplasts from *sh2* contain very little phosphoglucomutase or that it is inactive in the incubation conditions used.

Results of in vivo studies of the starch-deficient maize endosperm mutant *bt1* support the conclusion that BT1, an amyloplast-membrane-specific polypeptide (Cao et al., 1995; Sullivan and Kaneko, 1995), is an adenylate translocator that functions in ADP-Glc transfer into amyloplasts. For example, ADP-Glc, which is synthesized by AGPase, accumulates in the endosperm of *bt1* mutant kernels (Shannon et al., 1996). Activities of AGPase, UGPase, SS, extractable SBE, and SUS in extracts from *bt1* mutant endosperms were equal to or greater than activities in endosperm extracts from normal kernels (Shannon et al., 1996). The genetic lesion in *bt1* kernels was found to be an amyloplast-membrane-specific, 39- to 44-kD polypeptide, BT1 (Cao et al., 1995; Sullivan and Kaneko, 1995).

Based on these results, we suggest that BT1 is an adenylate translocator that functions in the transfer of ADP-Glc from the cytosol into the amyloplast, and in its absence (i.e. in *bt1* mutant kernels) ADP-Glc accumulates (Shannon et al., 1996). The most direct support for this suggestion is provided by the marked difference in the uptake of ADP-Glc and its use for starch synthesis by amyloplasts isolated from *bt1* endosperms and amyloplasts isolated from normal, *wx*, and *sh2* endosperms (Table V). Intact amyloplasts from *bt1* endosperms, which are missing the BT1 polypeptides, were only 26% as effective in taking up and converting ADP-Glc to starch as those from the other genotypes (Table V).

Several lines of evidence support the conclusion that we were measuring ADP-Glc uptake and utilization by intact amyloplasts and not simply synthesis by SS associated with granules released from lysed amyloplasts: (a) ADP-Glc incorporation by lysed amyloplasts was only about 10% of that by intact amyloplasts; (b) for many of the uptake and incorporation studies we used amyloplasts from the *wx* endosperm mutant, which is deficient in the starch-granule-bound starch synthase (Shannon and Garwood, 1984); and (c) the adenosine analog FSBA, which is known to react with adenosine-binding sites (Colman, 1983), more effectively inhibited uptake and incorporation of ADP-Glc than starch synthase (Table VIII).

Comparison of the translated full-length sequence of BT1 with protein sequences of 45 adenylate translocators from 20 species revealed about 30% identity and 81% similarity within the highly conserved regions of the mitochondrial adenylate translocators (Cao and Cao, 1997). If BT1 is an adenylate translocator, as was suggested, then the mature protein should contain an ADP-Glc-binding motif. However, no ADP-Glc-binding motif was present in the mature BT1 protein, assuming that the transit-peptide cleavage site VRA/A that was proposed by Sullivan et al. (1991) is correct. However, analysis of the full-length BT1 amino acid sequence showed the presence of the putative ADP-Glc-binding motif, KTGGL, 40 amino acid residues upstream of the cleavage site proposed by Sullivan et al. (1991) (Table VIII). For BT1 we propose an alternative transit-peptide cleavage site, VP/A, 13 amino acids upstream of the putative ADP-Glc-binding motif (Table IX). Transit-peptide cleavage at this site would yield a mature BT1 of 44 kD, which agrees well with the size we reported for mature BT1 (Cao et al., 1995) but is somewhat larger than the 39.5- and 38.5-kD mature BT1 polypeptides reported by Li et al. (1992).

In summary, we have provided evidence that most of the cellular AGPase in maize kernels in both the linear phase and in the early phase of starch accumulation resides in a compartment that partitions with cytosolic marker enzymes after nonaqueous and aqueous fractionation. However, based on the immunolocalization study of Brangeon et al. (1997), we suggest that in situ AGPase is functionally compartmented with the amyloplasts and may be loosely associated with the outer membrane of the amyloplast envelope. ADP-Glc is transported into the amyloplast stroma via BT1, which may be the same transporter as the ADP-Glc/AMP adenylate translocator described by Möhlmann et al. (1997). The importance of the BT1 translocator to starch accumulation in maize endosperms is demonstrated by the severely reduced starch content in *bt1* mutant kernels (Tobias et al., 1992). Assessment of the relative importance of the hexose-P/Pi antiporter for starch accumulation in vivo awaits isolation of a mutant genotype defective in the hexose-P/Pi antiporter.

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